

ORIGINAL PAPER

Higher immunoglobulin E antibody levels to recombinant Fel d 1 in cat-allergic children with asthma compared with rhinoconjunctivitis

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Clinical and Experimental Allergy

Summary

Background Current diagnosis of allergy and asthma to cat is confirmed using cat dander extract (CDE). We have previously engineered a recombinant major cat allergen, rFel d 1, with properties identical to the natural molecule.

Objective The aim of the study was to evaluate IgE and IgG4 antibodies to rFel d 1 among sera from cat-allergic children and adults suffering from asthma and/or rhinoconjunctivitis (RC) in populations from Sweden and Austria.

Methods Cat-allergic children and adults from Sweden ($n = 27$ and 31 , respectively) and Austria ($n = 41$ and 41) with RC and/or asthma were selected. Sera were tested for IgE and IgG4 antibodies to CDE and rFel d 1 by CAP, and IgE to rFel d 1 by ELISA. Healthy subjects and non-cat-allergic patients ($n = 75$) were included as controls.

Results There was a high correlation between IgE responses to rFel d 1 and CDE among the 140 patients ($r_s = 0.85$, $P < 0.001$); however, measured levels to rFel d 1 were on average 30% higher ($P < 0.0001$). Ninety-eight percent of patients and none of the controls showed IgE to rFel d 1 and there was a threefold increased risk of asthma for half of the children with the highest IgE levels [odds ratio 3.23; 95% confidence interval (CI), 1.19–8.79] by ELISA. IgE responses to rFel d 1 among children with asthma were higher (median 19.4 kU/L) compared with children with RC (median 6.6 kU/L, $P < 0.05$) and adults with asthma (median 3.0 kU/L, $P < 0.01$). Furthermore, children with asthma displayed higher IgG4 levels than the asthmatic adults.

Conclusion A single recombinant molecule, rFel d 1, is at least as sensitive for *in vitro* diagnostics of cat allergy as the current extract-based test. Elevated IgE antibody levels to Fel d 1 are suggested to be a risk factor for asthma in cat-allergic children.

Keywords asthma, cat, children, IgE, rFel d 1

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Introduction

Allergens derived from cats (*Felis domesticus*) are a common cause of IgE-mediated allergic disease all over the world [1, 2]. The symptoms range from relatively mild rhinoconjunctivitis (RC) to potentially life-threatening asthmatic exacerbations. In the past decades, a high incidence of allergy to cat, especially among children and young adults, has been recorded [3]. As shown in

several cohort studies, cat allergy involves about 10% of the population [4, 5] and is in some regions considered to be the primary cause of childhood asthma [6].

Cat dander extract (CDE) consists of several allergens. Beside Fel d 2 (albumin) [7], Fel d 3 (cystatin) [8] and Fel d 4 (lipocalin) [9], the major and most studied allergen is the 35-kDa secretoglobulin Fel d 1 [10–12]. This persistent and ubiquitously present allergen has been found in indoor dwellings such as in dust samples from homes with or

without cats [1, 13, 14] and in public buildings and public transportation [15, 16] making allergen avoidance difficult. Sensitization to Fel d 1 is reported in 65–95% of cat dander-sensitized individuals [17, 18], and accounts for most of the IgE antibodies to cat dander allergens [18, 19]. We recently described a recombinant chain 2 and 1 fusion protein that exhibits IgE-binding, structure and biological properties indistinguishable from natural (n) Fel d 1 [11, 12].

Despite several limitations, IgE measurements of cat allergy using crude allergen extracts have been available for several decades. Allergen extracts from natural source material are not consistent concerning quality and quantity of contained allergens [20, 21]. In the attempts to improve diagnostics, it has been suggested to replace natural extracts with sets of single purified recombinant molecules in order to obtain component-resolved diagnosis [22, 23], for example recombinant (r) Fel d 1 in the diagnosis of allergy to cat [24].

IgE antibodies to cat dander proteins are an important parameter of sensitization [25, 26]. Although the relationship between high IgE levels and genes associated with asthma has been established [27], yet not much is known about the quantitative relation of IgE to certain allergens in patients with asthma or RC only and in particular the antibody reactivity pattern to Fel d 1 in different regions of Europe and between children and adults. The role of allergen-specific IgG4 in allergic disease is debated. Antibodies of the IgG class, in particular IgG4, may offer protection by blocking IgE and have been suggested to reduce allergen presentation via the low-affinity IgE receptor on B cells [28, 29].

Here we report IgE and IgG4 antibody responses to a correctly folded r Fel d 1 protein using sera from Swedish and Austrian children and adults with cat-induced allergic asthma or RC as measured by ImmunoCAP System and ELISA.

Material and methods

Study population

Sixty-eight children with a positive skin prick test (SPT) (weal size > 3 mm) to cat extract (ALK, Horsholm, Denmark) were enrolled in the study with their parents' consent. Venous blood was drawn and serum was prepared from all participating children. Twenty-seven children were recruited from Astrid Lindgren Children's Hospital, Stockholm, Sweden, and 41 from the Department of Pediatrics, Medical University of Graz, Austria (Table 1). All patients had a diagnosis of RC and/or asthma induced by cat. The patients were divided into two groups: asthma with or without RC (asthma) or RC only. None of the patients had previously received immunotherapy to cat. For control purposes, age-matched serum samples were obtained from 25 non-allergic children (Department of Pediatrics, Medical University of Vienna, Austria) and from

Table 1. Demographic data at inclusion

	All included	Sweden	Austria
Cat-allergic children			
No. of patients	68	27	41
Age (year): mean (range)	11.2 (2–17)	11.6 (5–17)	11.0 (2–17)
Sex: male/female	46/22	16/11	30/11
Asthma/RC	34/33	15/12	19/21
Cat-allergic adults			
No. of patients	72	31	41
Age (year): mean (range)	36.8 (20–60)	34.3 (20–60)	38.6 (26–64)
Sex: male/female	26/46	14/17	12/29
Asthma/RC	38/34	21/10	17/24
Non-cat-sensitized children			
No. of patients	50	25	25
Age (year): mean (range)	10.0 (3–17)	10.5 (5–17)	9.6 (3–16)
Sex: male/female	26/24	15/10	11/14
Non-cat-allergic adults			
No. of patients	25	14	11
Age (year): mean (range)	29.9 (19–45)	30.5 (19–45)	29.1 (23–39)
Sex: male/female	15/10	9/5	6/5

25 children whose serum samples were submitted for routine *in vitro* allergy diagnostics at Karolinska University Hospital (KUH). All lacked IgE antibodies (<0.35 kU_A/L) for CDE (UniCAP, Phadia AB, Uppsala, Sweden).

Using the same inclusion criteria, 31 and 41 cat-allergic adults were recruited from Sweden and Austria, respectively (KUH/Danderyd Hospital, Stockholm, Sweden and Medical University of Vienna, Austria).

Twenty-five adult patients diagnosed with allergy to other allergens (e.g. pollen and mites) but not to cat were included as adult controls. The study was approved by the local ethics committees.

Recombinant Fel d 1

rFel d 1 was produced as described [11]. Briefly, rFel d 1 [30] representing a chain 2–chain 1 fusion protein was expressed as folded protein in *Escherichia coli* and purified to homogeneity [12]. The proper folding of the rFel d 1 has been demonstrated previously by inhibition ELISA, basophil stimulation, CD spectrum and X-ray crystallography [11, 12]. The protein was stored at –70 °C until use.

Antibody measurement by ImmunoCAP

rFel d 1 was covalently bound to the CAP solid support (kindly supplied by MIAB, Uppsala, Sweden). CDE CAPs (e1) were purchased from Phadia AB. Quantitative IgE and IgG4 antibody responses to rFel d 1 and CDE in sera from cat-allergic patients were analysed by ImmunoCAP System (Phadia AB) using the cut-off limits of 0.35 kU_A/L and 150 µg/L, respectively, as recommended by the manufacturer. Total

IgE levels were measured by the ImmunoCAP System (Phadia AB) among children from Sweden and Austria ($n = 23$ and 33 , respectively) and adults ($n = 30$ and 41 , respectively). The amount of coupled Fel d 1 to ImmunoCAP was estimated using the ImmunoCAP System. The percent increase of IgE responses of rFel d 1 compared with CDE CAP across the cohort was calculated as medial values (rFel d 1-CDE)/CDE $\times 100$. Monoclonal anti-Fel d 1 antibody (MAb 2463, Phadia AB) diluted to 0.1, 0.3, 1.0, 3.0 and 7.0 $\mu\text{g/mL}$, and corresponding anti-mouse IgG ImmunoCAP was used to generate a reference curve. CDE and rFel d 1 ImmunoCAP was analysed by probing with 2.0 $\mu\text{g/mL}$ of anti-Fel d 1 MAb, followed by an anti-mouse IgG- β -galactosidase conjugate (Phadia AB).

Immunoglobulin E antibody measurement to recombinant Fel d 1 by enzyme-linked immunosorbent assay

Analyses of IgE antibodies to rFel d 1 were performed by ELISA using 96-well micro-titre plates. Briefly, wells were coated with 0.5 μg rFel d 1, followed by bovine serum albumin blocking. Diluted serum (1:2) was added in duplicates, followed by rabbit anti-human IgE (MIAB), goat anti-rabbit IgG-alkaline phosphatase conjugate (Dako, Glostrup, Denmark) and substrate [11].

For comparison between rFel d 1 ELISA plates, a negative and a positive reference serum pool were prepared, each containing serum from five adult patients. Values were considered to be positive when they exceeded the cut-off, defined as $+3$ SD of the mean of the respective control groups (Table 1).

Statistical analysis

The Mann-Whitney U -test was used to calculate differences between groups, Wilcoxon' matched pairs test for comparison

of IgE responses to rFel d 1 and CDE CAP and Spearman Rank correlation analyses were performed to determine correlation coefficients. Logistic regression was used to evaluate the risk of asthma in children with elevated levels of IgE to rFel d 1. $P < 0.05$ was considered to be statistically significant.

Results

Immunoglobulin E analyses of sera using ImmunoCAP

Positive allergen-specific IgE antibody responses were recorded in 65 out of 68 (95.6%) cat-allergic children and in 68 out of 72 (94.4%) adults using rFel d 1 CAP. Similarly, 64 children and 71 adults were positive by CDE CAP (Fig. 1a, Table 2).

No difference in IgE antibody levels to cat allergens was noted between children enrolled in Sweden and Austria ($P > 0.05$; Figs 1a and b). On the other hand, when children from each country were analysed separately or combined, they displayed significantly higher median IgE levels to both CDE and rFel d 1 than the group of 72 cat-allergic adult patients (Table 2). A high correlation between rFel d 1 and the CDE CAPs among the 140 patients was demonstrated ($r_s = 0.85$, $P < 0.001$; Fig. 2). However, the measured IgE responses to rFel d 1 were on average 30% higher than to the CDE ($P < 0.0001$).

No difference in total IgE levels was observed between the Swedish and Austrian children or between Swedish and Austrian adults ($P > 0.05$), but children had significantly higher total IgE compared with adults ($P < 0.01$; Table 2). However, no difference was noted between children with asthma and children with RC or between adults with asthma and adults with RC ($P > 0.05$). The ratio of Fel d 1-specific IgE to total IgE did not differ between children with asthma and RC ($P = 0.93$).

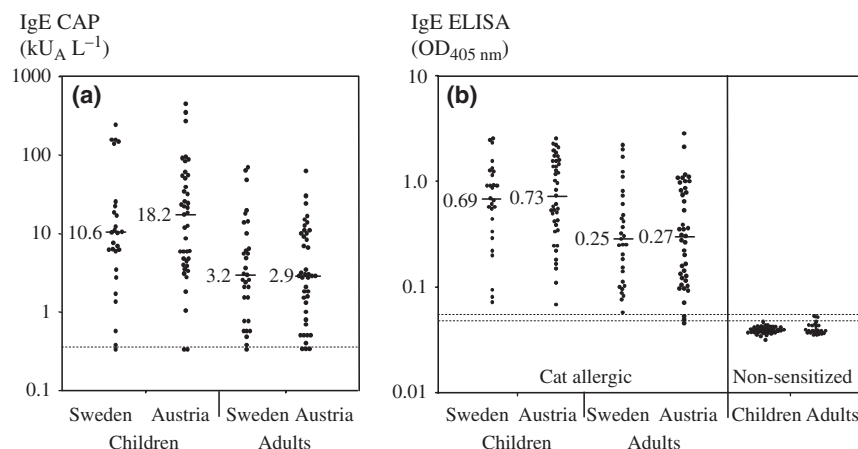
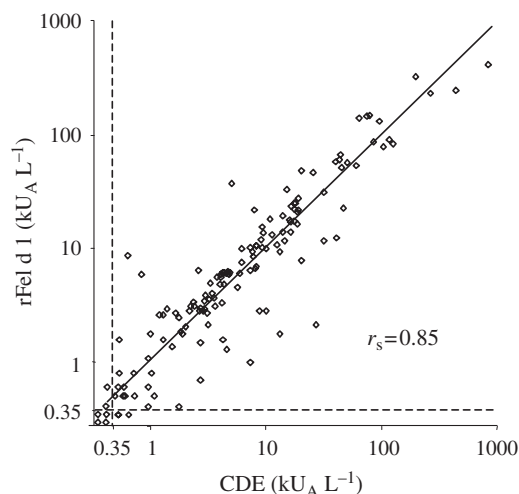


Fig. 1. IgE responses to rFel d 1 in cat-allergic children from Sweden ($n = 27$) and Austria ($n = 41$) and adults from Sweden ($n = 31$) and Austria ($n = 41$) analysed by (a) CAP System and (b) ELISA. The hatched bar denotes the 0.35 kU_A/L cut-off (a), and the upper and lower hatched bars denote $+3$ SD of the mean for adult control subjects ($n = 25$) and control children, respectively ($n = 50$) (b).

Table 2. Comparison of antibody responses in serum from cat-allergic Swedish and Austrian children ($n = 27$ and 41 , respectively) and adult patients ($n = 31$ and $n = 41$, respectively)

Cat allergen and test system	Ab*	Children		Adults		P -value [†] (children vs. adults)
		Sweden	Austria	Sweden	Austria	
		Median, range	Median, range	Median, range	Median, range	
CDE, CAP [‡]	IgE	6.7, <0.35–230	12.8, <0.35–730	2.3, <0.35–38.0	3.5, 0.42–43.7	<0.001
rFel d 1, CAP [‡]	IgE	10.6, <0.35–258	18.2, <0.35–473	3.2, <0.35–73.4	2.9, <0.35–62.0	<0.001
rFel d 1, ELISA [§]	IgE	0.69, 0.071–2.50	0.73, 0.067–2.20	0.25, 0.057–2.19	0.27, 0.049–2.62	<0.001
rFel d 1, CAP [¶]	IgG4	665, <150–7200	360, <150–7380	190, <150–5700	155, <150–5660	>0.05
Total IgE CAP	IgE	280, 48–1900	460, 70–30 200	133, 15–8970	210, 27–2700	<0.01

*Allergen-specific antibodies.

[†]Mann-Whitney U -test.[‡]Allergen-specific antibodies measured in kU_A/L.[§]Allergen-specific antibodies measured in optical density at 405 nm.[¶]Allergen-specific antibodies measured in µg/L.^{||}Allergen-specific antibodies measured in kU/L.**Fig. 2.** Spearman Rank analyses of IgE responses to CDE CAP (x-axis) and rFel d 1 CAP (y-axis) in 140 patients. Marked lines denote the cut-off levels (0.35 kU/L).

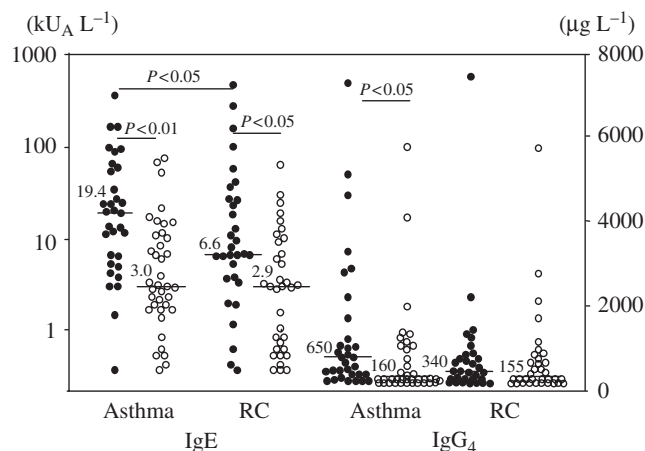
Analysis of immunoglobulin E antibodies to recombinant Fel d 1 by enzyme-linked immunosorbent assay

Sera from the cat-allergic patients were analysed by ELISA. All 68 children and 69 of 72 (95.8%) adults showed IgE levels to rFel d 1 exceeding the cut-off limit (Fig. 1b).

The IgE reactivity by the rFel d 1 ELISA in comparison with rFel d 1 CAP and CDE CAP was closely related. The correlation coefficient (r_s) among the 140 patients was 0.94 and 0.89, respectively ($P < 0.001$).

Analysis of immunoglobulin G4 antibodies to recombinant Fel d 1 by ImmunoCAP

The concentration of naturally occurring IgG4 antibodies to rFel d 1 in the 72 cat-allergic adults and, due to

**Fig. 3.** Levels of IgE (kU_A/L) and IgG4 (µg/L) antibodies to rFel d 1 (y-axis) in children (●) with asthma ($n = 34$) or rhinoconjunctivitis (RC) ($n = 33$) to cat. Seventy-two cat-allergic adults (○) diagnosed with asthma ($n = 38$) or with RC ($n = 34$) were used for comparison.

shortage of sera, in 63 out of the 68 cat-allergic children (Table 2) was analysed by CAP. In 20/23 (87%) and 33/40 (83%) of the children from Sweden and Austria, respectively, and in 42/72 (58%) of the adults the serum IgG4 concentrations exceeded the cut-off limit (Fig. 3).

In contrast to IgE, no difference in the IgG4 levels to rFel d 1 was detected between the children and the adult patient groups. Furthermore, the IgE and IgG4 levels in children exhibited a weak correlation ($r_s = 0.44$, $P < 0.001$; Fig. 4) whereas no correlation was noted among the adult patients ($r_s = 0.05$, $P > 0.05$). Sera from non-sensitized children ($n = 40$) and adults ($n = 21$) were analysed for IgG4 antibodies to rFel d 1. Medians for both groups were below the cut-off; 11/40 (28%) of the children and 5/21 (24%) of the adults (range 150–6250 and 150–935 µg/L, respectively) were positive.

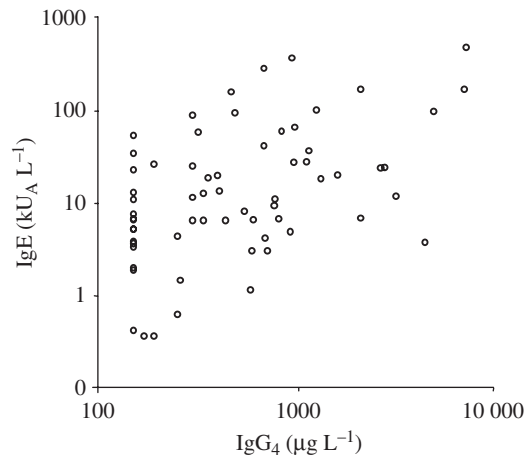


Fig. 4. Spearman Rank analyses of IgG4 (x-axis) and IgE (y-axis) responses to rFel d 1 by CAP in 65 cat-allergic children.

Analysis of Fel d 1 bound to ImmunoCAP

The amount, quality and ligand density of allergens on the solid phase are critical for the overall performance of an allergy diagnostic device. Therefore, the relative amount of Fel d 1 in ImmunoCAP was analysed using a monoclonal anti-Fel d 1 antibody. The respective antibody binding to CDE and rFel d 1 corresponded to 0.6 and 2.0 µg/mL (CV range 1.8–3.4%), showing a 3.3 times higher concentration in the rFel d 1 CAP.

Antibody responses to cat allergens in relation to asthma and rhinoconjunctivitis

Among children from Sweden and Austria, 15 of 27 (56%) and 19 of 40 (48%), respectively, were diagnosed with asthma in contact with cats. In the adult group, 21 of 31 (68%) Swedish and 18 of 41 (44%) Austrian patients had asthma to cat.

No difference in IgE levels to CDE and rFel d 1 was observed between Swedish and Austrian children; however, the IgE concentrations to rFel d 1 were significantly higher in asthmatic children ($n=34$) compared with children with RC only ($n=33$) (Fig. 3). Furthermore, children with asthma or RC displayed significantly higher concentrations of IgE to Fel d 1 (median 19.4 and 6.6 kU/L) than comparable adults (IgE median 3.0 and 2.9 kU/L). Analysis of IgG4 showed that children with asthma had higher levels (median 650 µg/L, $P<0.05$) compared with adults with asthma (median 160 µg/L). In contrast to the children, no difference between adult patients with asthma or RC regarding rFel d 1 antibody levels was detected (IgE and IgG4, $P>0.05$) (Fig. 3).

Discussion

At first glance, IgE antibodies induced by an allergen source may be best recognized by antigens from the

source itself and therefore are superior diagnostic tools compared with man-made allergens. However, this assumption can be disputed because natural allergen sources vary unpredictably in the quality as well as quantity of individual allergens and other molecules. On the other hand, allergens engineered and produced by recombinant techniques may provide an unlimited source of pure and well-defined proteins for immunological investigations, diagnostic and therapeutic purposes. We show here that a correctly folded single recombinant allergen, Fel d 1, equals CDE when diagnosing cat-allergic patients by CAP. Furthermore, we report that cat-allergic children have higher IgE levels to rFel d 1 and higher total IgE levels compared with adults, suggesting that adults generally have a less active immune system or a more suppressed IgE production over time. This is in line with the observation that allergic diseases seem to decline with age [31].

IgE measurements using CDE are well established in patients allergic to cat. Moreover, it has been shown that allergen-specific IgE levels are predictive for the likelihood of allergic disease [32, 33]. Ninety-six percent of sera from the cat-allergic patients showed positive IgE levels to CDE. Yet, the single and structurally well-characterized rFel d 1 bound to CAP recognized all CDE-positive patients except one. Unexpectedly, the IgE levels recorded in the 140 patients by CAP to the purified rFel d 1 molecule were significantly higher compared with the commercially available CDE CAP. This may be due to the approximately three times lower levels of natural Fel d 1 on the CDE ImmunoCAP compared with the rFel d 1 ImmunoCAP. Consequently, we found on average 30% in the combined cohort and in the particular sera 10 times higher IgE response to the single rFel d 1 CAP compared with CDE CAP without loss of specificity. It is likely that molecules other than rFel d 1 in the extract compete for space on the solid phase, thus allowing suboptimal quantitation of allergen-specific IgE by extract-based diagnostics.

Ohman and Lowell [17] have reported previously that 65% of cat-allergic patients were sensitized to Fel d 1. More recently, 96% of sera, pre-selected on the basis of IgE antibodies to CDE, were reported to be positive [18] to a not fully refolded mix of the two Fel d 1 chains [34]. In our study, where the patients were selected on the basis of doctors' diagnosis of cat allergy, we demonstrated that IgE responses to rFel d 1 analysed by ELISA resulted in an even higher sensitivity and specificity (98% and 100%, respectively). It should be noted that the study design did not include persons with sensitization but without symptoms, which potentially may reduce the diagnostic sensitivity and, furthermore, that the cut-off used for the ELISA was calibrated to this particular allergen based on the control group. The cut-off recommended by the manufacturer of ImmunoCAP, 0.35 kU/L, is universal for all

allergens, extracts of various sources as well as single allergen components. Adaptation of cut-off to a particular allergen may therefore improve the sensitivity of the test. The finding that single allergens may prove more sensitive than the combination of allergens in an extract may not be applicable to complex allergen sources. Although measurement of additional single allergens (e.g. cat allergens Fel d 2–6) may improve the sensitivity, single allergen analysis will likely be important in the development of new and clinically useful diagnostic procedures.

Sensitization to allergens, and in particular to cat dander, is assumed to be a risk factor for development of asthma [35]. In our study, more than 50% of the cat-sensitized patients had a diagnosis of asthma. We found that the group of children with asthma or RC had significantly higher IgE levels to rFel d 1 compared with the respective groups of adults. In contrast to adults, we observed that children with asthma had higher rFel d 1-specific IgE levels compared with children with RC only. Thus, high IgE levels to rFel d 1 among children seem to be associated with an increased risk of asthma, either as a secondary phenomenon or as a pathogenic factor. This is in accordance with a recent study by Simpson *et al.* [36] showing that the risk of current wheeze increased significantly with increasing IgE levels to CDE. Interestingly, the IgE levels to rFel d 1 were similar among children and adults from northern and central Europe, suggesting no major difference of allergen exposure.

However, neither the total IgE level nor the relative ratio of Fel d 1-specific IgE to total IgE was higher among asthmatic children compared with children with RC. This could suggest that no polyclonal up-regulation of total IgE levels has occurred, but rather that the IgE to rFel d 1 by itself may be indicative for development of asthma.

Similar to IgE, the IgG4 antibody levels to Fel d 1 were significantly higher in children with asthma compared with adults, suggesting a stronger activation of T-helper type 2-driven events. This enhanced activation thus seems to contribute to a more severe allergic disease. The results are in line with the recent studies from the ALEX population where the prevalence of allergic disease was higher in children expressing both IgG4 and IgE [37].

Important findings in this study were, first, in agreement with the diagnosis, 98% of cat-allergic patients displayed IgE antibodies to rFel d 1 by a sensitive ELISA compared with 96% using CDE CAP, suggesting that a single recombinant allergen molecule may be as good as or even superior compared with the combination of natural allergens in an extract source. The diagnostic value was also emphasized by the significantly higher IgE binding to rFel d 1 CAP compared with CDE CAP. Second, children with asthma due to cat allergy, but not adults, exhibited significantly higher IgE responses to Fel d 1 than patients with RC only, suggesting increased Fel d

1-specific IgE levels as a potential risk factor for allergic asthma in children.

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